Office of Naval Research
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I. SELECTIVE SORPTION OF BACTERIA FROM SEAWATER

II. MECHANISM OF THE INITIAL EVENTS IN THE SORPTION OF MARINE BACTERIA TO SURFACES

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SELECTIVE SORPTION OF BACTERIA FROM SEAWATER

by

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Summary

A distinct sequence of sorption of different bacterial types has been observed both on glass slides and electron microscope grids immersed in seawater for periods of up to 24 hours. A comparison of the bacterial groups initially attracted to a surface with those subsequently adhering firmly to the surface suggests a selective irreversible sorption of certain groups of marine bacteria. Firm adhesion of bacteria in the short time periods considered does not involve pili or holdfast structures. The ability to produce extracellular polymeric fibrils may be important in such selective sorption.

Introduction

The formation of primary microbial films on surfaces immersed in seawater is believed by many investigators to be a prerequisite to fouling by

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barnacles and other invertebrate larvae (see Wood, 1967; Corpe, 1970a). Such films have been variously described as being predominantly bacterial, algal or diatom films, but these observations have been made on surfaces immersed in seawater for several days or even much longer periods.

It is important to obtain information on the organisms responsible for the primary colonization of surfaces, since these primary colonizers probably modify the surface properties of the material immersed in the seawater. Any modification to the surface may render it more amenable to colonization by other groups of microorganisms. Implicit in this proposal is that certain bacteria are selectively sorbed, and that by modification of the sorption surface a distinct succession of types sorbing might be expected. Zvyagintsev (1959) has noted the selective sorption to glass surfaces of soil bacteria.

The aim of the present investigation was to determine if selective sorption of bacteria occurs at short times of immersion of surfaces in seawater, and whether specialized attachment appendages were advantageous to bacteria initially adhering to a surface.

Methods

Sorption to glass surfaces: Slides were immersed in seawater samples under laboratory conditions at 25°, the water being stirred slowly with a magnetic stirrer. At regular intervals, duplicate slides were removed from the water, washed several times with 2.5°/o NaCl, and examined under a phase-contrast microscope. Where detailed counts were made the bacteria were divided into 5 categories: A, small rods (less than 0.8μ long); B, large rods (greater than 0.8μ long); C, cocco-bacilli; D, curved rods (vibrio and spiral forms); E, stalked bacteria (caulobacters and hyphomicrobia).

At least 10 random fields were counted on each of the duplicate slides at each sampling period.

Studies on the sorption of pure cultures to surfaces (Marshall, Stout and Mitchell, 1970) have indicated two distinct phases in the sorption process which may be described as the reversible and irreversible phases. A comparison has been made of the population of cells initially attracted to a glass surface (reversibly) with that finally adhering firmly to the surface (irreversibly). Reversible sorption was examined by running small samples of seawater under glass coverslips supported on slides by broken pieces of coverslips. The samples were allowed to stand for 15 to 30 min. prior to microscopic examination. This allowed those cells not attracted to the coverslip surface to fall away from the field of view by gravitational force. Counts were made of the 5 categories of bacteria detailed above. Irreversible sorption was determined on slides immersed in the seawater samples for 24 hrs., prior to rinsing with 2.5°/o NaCl. The seawater samples were collected at different times and stored under a variety of conditions.

Sorption to electron microscope grids: Nickel grids with collodion-carbon films were immersed directly in the sea at Winthrop, Massachusetts or in freshly collected seawater samples in the laboratory. In some instances, grids were inserted in lucite boxes designed to prevent bacteria at the air-water interface from sorbing to the grids. This precaution was not necessary, since such bacteria did not firmly sorb to surfaces and were washed off in subsequent treatments. At each sampling time, duplicate grids were removed, fixed in 2.5°/o formaldehyde in 2.5°/o NaCl for 30 min. (Hodgkiss and Shewan, 1968), dried, rinsed with distilled water to remove soluble salts, and dried

prior to negative staining with phosphotungstate (Bradley, 1962) or shadowing with gold-palladium (60^{o}/o gold:40^{o}/o palladium).

Results

Electron microscope observations

Bacteria were found on grids after one hr. immersion in seawater, and numbers on the grids increased with increasing immersion time. Regardless of whether the grids were immersed in the sea or in seawater under laboratory conditions, the predominant cells were conventional rod-shaped bacteria. Most cells had rough surface features (Pl. 1, figs. 1 and 2) and often possessed distinct electron-dense polar bodies (Pl. 1, fig. 3). Many cells were extremely small (Pl. 1, fig. 4), while some curved (Pl. 1, fig. 5) and spiral (Pl. 1, fig. 6) cells were found.

Specialized attachment appendages (pili, holdfasts) were not found on most bacteria sorbing in periods of up to 24 hrs. Some bacteria produced extracellular polymeric strands that might be of significance in the irreversible sorption of the cells to surfaces (Pl. 2, figs. 7 and 8). After 24 hrs. immersion, a few stalked bacteria, mostly hyphomicrobia, were found (Pl. 2, fig. 9). Individual pear-shaped cells resembling "swarmer" cells of the hyphomicrobia sometimes were observed (Pl. 2, fig. 10). This suggests that such swarmer cells may sorb rapidly, but that growth of stalks and buds is not obvious until much later.

Sequence of sorption on glass surfaces

Preliminary examination of slides immersed in seawater indicated that some rod-shaped bacteria were sorbed within one hr. Coccoidal and spiral

forms generally were observed after 6 to 8 hrs. Some stalked bacteria were found after 24 hrs. and constituted a significant fraction of the population after several days.

A more detailed estimate of the numbers of different groups of bacteria sorbed to glass surfaces up to 24 hrs. is presented in Fig. 1. Stalked bacteria were not observed during the period of the test. The total numbers of the other categories generally increased with increasing time, the small rods being predominant at all sampling times. However, the proportion of small rods in the total sorbed population decreased at 24 hrs., while the proportions of the large and curved rods increased (Fig. 2). This result suggests that small rods are rapidly sorbed following immersion of slides in the seawater. The rates of sorption of the other types are slower, and may be favoured by prior sorption of the small rods.

Selectivity of bacterial sorption

The results presented above do not indicate whether selection in the sorption process is in the initial attraction of cells to a surface or in the subsequent firm adhesion phase. In a comparison of bacterial types reversibly and irreversibly sorbed from a range of different seawater samples, some consistent trends in the selective colonization of surfaces were observed (Fig. 3). The results show the percentage of cells in each category irreversibly sorbed plotted against the percentage reversibly sorbed. Selectivity in the firm adhesion of bacteria to the glass surface is shown by any point above the diagonal line in Fig. 3. It is obvious that rod-shaped bacteria predominate in these different seawater samples, and that there is a selective irreversible sorption of the short rods. A more random spread of points is indicated for the curved rods and cocco-bacilli, suggesting that any selective

sorption of each types probably varies with the actual species present and the prevailing conditions in the particular water sample.

Stalked bacteria were rarely observed. It is possible, of course, that any swarmer cells (see Pl. 2, Fig. 10) of stalked bacteria may have been included in other categories because of the absence of any stalks.

Discussion

The results presented suggest that the mere attraction of marine bacteria to a glass surface does not guarantee that all of the bacteria will firmly adhere to the surface. It appears that the group designated as small rods possesses some selective advantage over other bacterial groups in the irreversible sorption to surfaces. This advantage might result from a superior ability to metabolize and multiply in the low nutrient environment of natural seawater, or it might be related to extracellular polymeric fibril production (Friedman, Dugan, Pfister and Remsen, 1969). The significance of growth and polymer production in the sorption process is considered in detail elsewhere (Marshall, Stout and Mitchell, 1970). Although pili have been observed on marine bacteria (Hodgkiss and Shewan, 1968), it is unlikely that such appendages are of significance in those bacteria responsible for the primary colonization of surfaces.

The apparent succession of bacterial types colonizing a surface may suggest an alteration of the test surface by those bacteria initially sorbed. This could result from the release of nutrients, surface active agents or extracellular polymeric materials (Corpe, 1970b). Under such conditions, those microorganisms sorbing to a surface after the initial colonization by other bacteria may be adhering to a very different surface to that encountered

by the initial group of bacteria.

Stalked bacteria have been reported on surfaces in freshwater environments (Henrici and Johnson, 1935; Tyler and Marshall, 1967). Corpe (1970a) has noted large numbers of stalked bacteria on slides immersed in seawater. The results reported here confirm Corpe's observations that such bacteria are a regular component of microbial surface films formed in marine environments. However, stalked bacteria may not be responsible for the initial colonization of surfaces.

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Explanation of Plates

Plate 1

Figure 1. Rough surface features of bacterium on grid immersed in sea at Winthrop for 6 hrs. Negatively stained.

Figure 2. As for Figure 1, but grid immersed for 2 hrs.

Figure 3. Long rod with prominent electron-dense polar bodies. Grid immersed for 3 hrs. in Woods Hole seawater in the laboratory. Unshadowed.

Figure 4. Very small coccoidal cell on grid immersed in ea at Winthrop for 1 hr. Negatively stained.

Figure 5. Group of curved rods with prominent surface features. Grid immersed for 24 hrs. in Nahant seawater in the laboratory. Negatively stained.

Figure 6. Spiral bacterium on grid immersed in sea at Winthrop for 1 hr. Shadowed.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 6

Fig. 5

Plate 2

Figure 7. Rods with extracellular polymeric fibrils and distinct polar bodies. Grid immersed in Woods Hole seawater in the laboratory for 1 hr. Shadowed.

Figure 8. Group of cells showing polymeric fibrils binding cells to surface and to each other. Grid immersed in Nahant seawater in the laboratory for 24 hrs. Shadowed.

Figure 9. Pear-shaped, stalked hyphomicrobium and rod-shaped bacterium. Grid immersed in Nahant seawater in the laboratory for 24 hrs. Shadowed.

Figure 10. Pear-shaped cell resembling swarmer of hyphomicrobium. Grid immersed in sea at Winthrop for 1 hr.

PLATE II

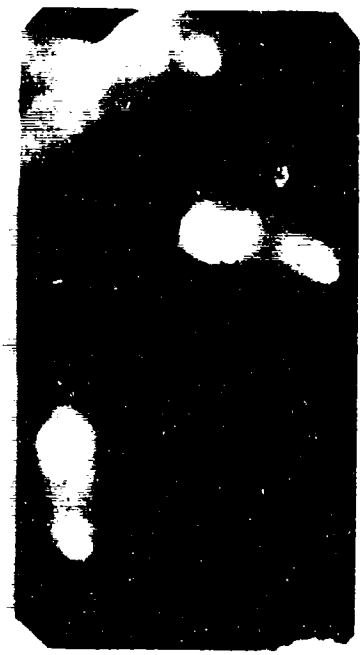

Fig. 7

Fig. 8

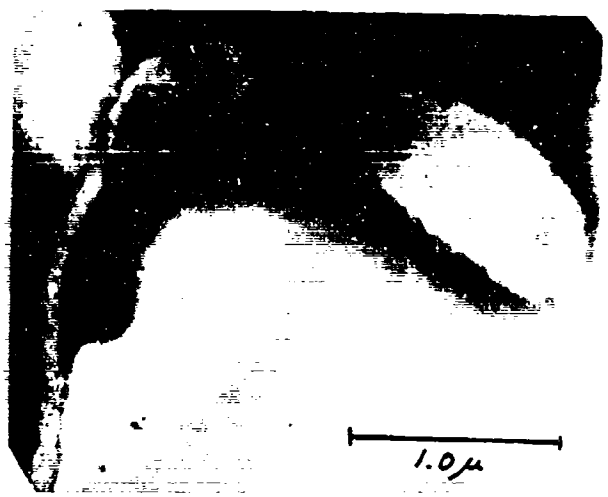

Fig. 9

Fig. 10

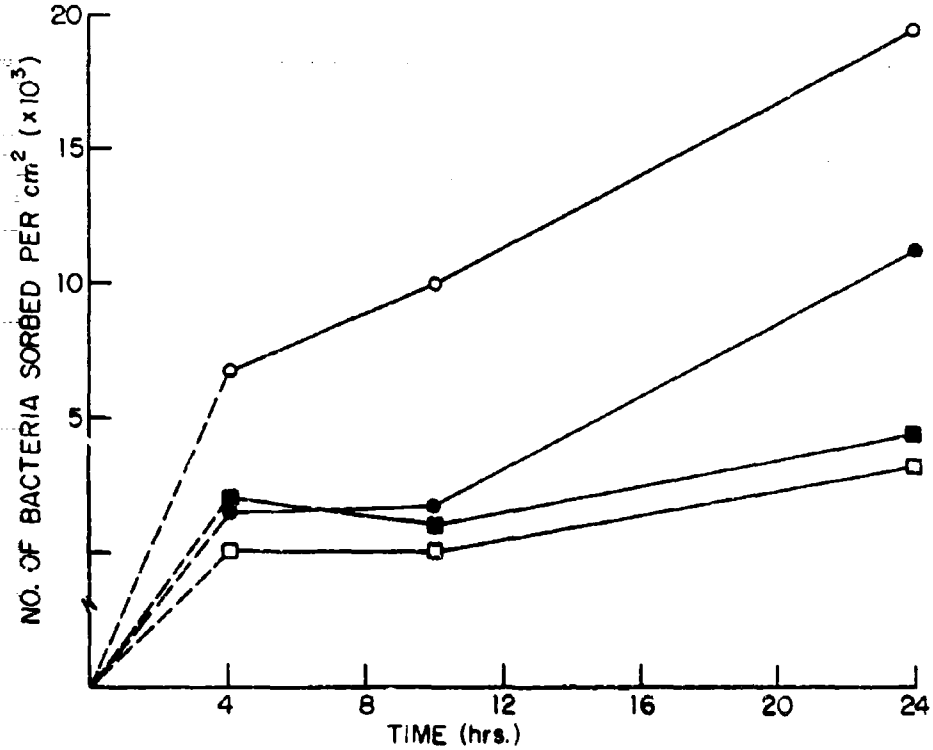

Figure 1. Numbers of various groups of bacteria irreversibly sorbed to glass slides immersed in seawater in the laboratory. O—O, small rods; ●—●, large rods; ■—■, cocco-bacilli; □—□, curved rods. Stalked bacteria not observed.

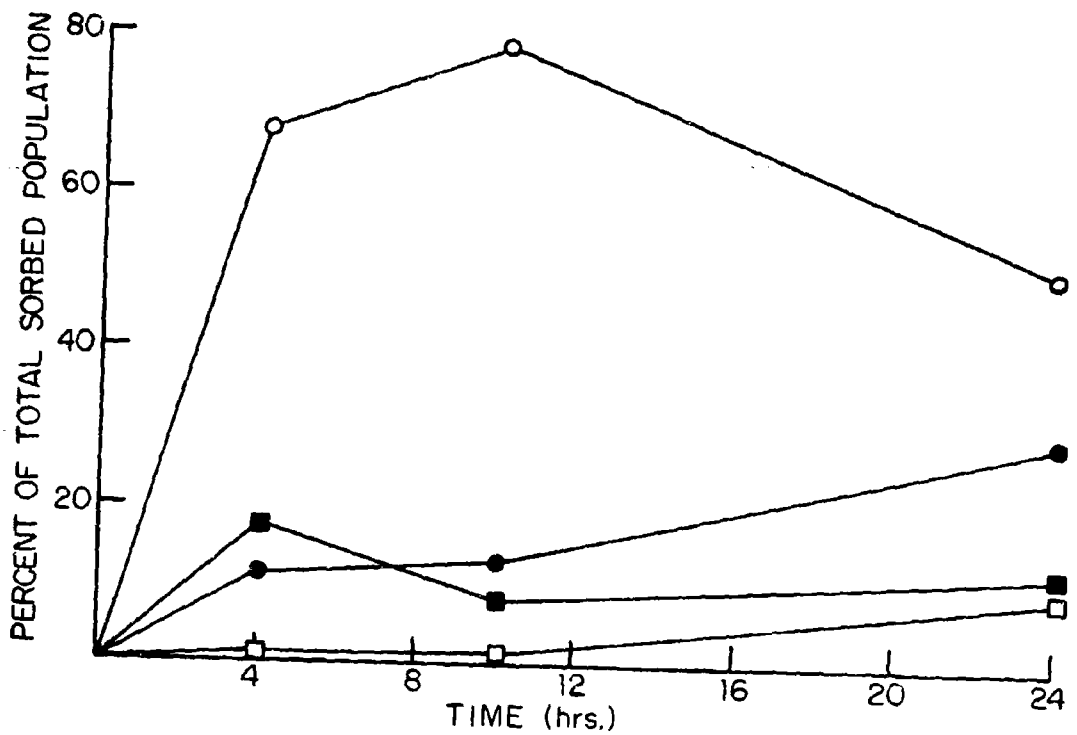

Figure 2. Various groups of bacteria irreversibly sorbed to glass slides expressed as a percentage of the total number sorbed at each sampling time. Symbols as in Figure 1.

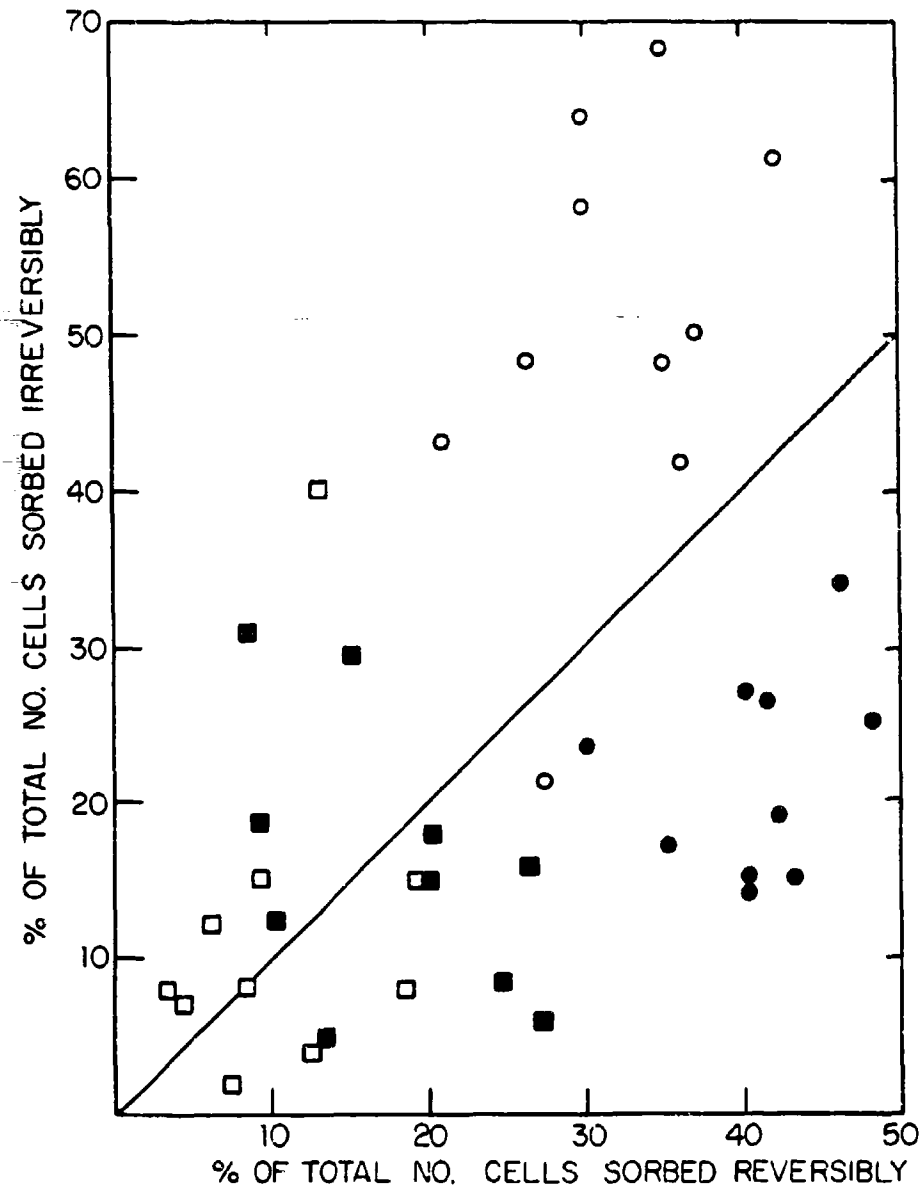

Figure 3. Selective sorption of bacteria from 10 different seawater samples. A comparison of the percentage of bacterial types reversibly sorbed with those irreversibly sorbed. Symbols as in Figure 1.

References

Bradley, D. E. (1962) A study of the negative staining process. Journal of General Microbiology, 29, 503.

Corpe, W. A. (1970a) Attachment of marine bacteria to solid surfaces. In Biological Adhesions, Ed. by Manly, P., New York, Academic Press.

Corpe, W. A. (1970b) An acid polysaccharide produced by a primary film-forming marine bacterium. Developments in Industrial Microbiology, 11, 402.

Friedman, B. A., Dugan, P. R., Pfister, R. M. and Remsen, C. C. (1969) Structure of exocellular polymers and their relationship to bacterial flocculation. Journal of Bacteriology, 98, 1328.

Henrici, A. T. and Johnson, D. E. (1935). Studies on fresh-water bacteria II. Stalked bacteria, a new order of the Schizomycetes. Journal of Bacteriology, 30, 61

Hodgkiss, W. and Shewan, J. M. (1968) Problems and modern principles in the taxonomy of marine bacteria, Advances in Microbiology of the Sea, 1, 127.

Marshall, K. C., Stout, R. and Mitchell, R.(1970) Mechanism of the initial events in the sorption of marine bacteria to surfaces. Journal of General Microbiology (submitted for publication).

Tyler, P. and Marshall, K. C. (1967) Form and function in manganese oxidizing bacteria. Archiv für Mikrobiologie, 56, 344.

Wood, E. J. F. (1967) Microbiology of Oceans and Estuaries, Amsterdam, Elsevier Publishing Co.

Zvyagintsev, D. G. (1959)
Microbiology (USSR) (English Translation), 28, 104.

REVERSIBILITY OF THE INITIAL EVENTS IN THE SORPTION OF MARINE BACTERIA TO SURFACES

by

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Summary

Two phases in the process of sorption of marine bacteria to surfaces have been defined as (a) an instantaneous reversible phase, and (b) a time-dependent irreversible phase.

Reversible sorption of the non-motile Achromobacter sp. strain R8 decreases to zero as the electrolyte concentration decreases, or as the thickness of the electrical double-layer increases. The electrolyte concentration at which all bacteria are repelled from the glass surface depends on the valency of the electrolyte. The reversible phase of sorption is interpreted in terms of the balance between the electrical double-layer repulsion energies at different electrolyte concentrations and the van der Waals attractive energies. Even at the electrolyte concentration of seawater, the bacteria probably are held at a small but finite distance from the glass surface by a repulsion barrier. The rotational motion of the motile Pseudomonas sp. strain R3 at a liquid-glass interface in considered in terms of the concept of reversible sorption.

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It is proposed that irreversible sorption results from the repulsion barrier being overcome by polymeric bridging between the bacterial and glass surfaces. Evidence is presented for the production of polymeric fibrils by Pseudomonas R3 in an artificial seawater. Sorption and polymer production are stimulated by very low levels of glucose (7 mg/l), but higher levels of glucose inhibited irreversible sorption. Deletion of both Ca^{2+} and Mg^{2+} from the artificial seawater prevented growth, polymer production, and sorption to surfaces by Pseudomonas R3.

Introduction

The sorption of bacteria to surfaces is a general phenomenon encountered in natural environments with important ecological implications (Wood, 1967; Marshall, 1970). Primary microbial film formation on surfaces immersed in seawater is considered by some investigators to be a prerequisite to fouling by larger organisms such as barnacles (Wood, 1967). The mechanism whereby marine bacteria sorb to surfaces has received scant attention. ZoBell (1943) suggested that once bacteria are attracted to a surface firm attachment requires an incubation period of several hours. He attributed this delay to the need for the synthesis of extracellular adhesive materials. Recently, Corpe (1970a) has reported the production of an extracellular acid polysaccharide by a primary film-forming bacterium, Pseudomonas atlantica. Glass slides coated with this polymer became fouled with microorganisms more rapidly than uncoated slides. Corpe (1970b) has reviewed the current literature on the attachment of bacteria to surfaces immersed in marine environments.

Since the average marine bacteria are almost of colloidal size, the aim of the present investigation was to combine a study of some of the colloidal and biological properties of pure cultures of marine bacteria to obtain

information on processes involved in the sorption of such bacteria to surfaces.

Definition of Phases of Sorption

Our studies have confirmed ZoBell's (1943) suggestion that sorption consists of two phases. The bacteria first were attracted to a surface, and, after a period of several hours, some bacteria become firmly attached to the surface. For convenience these phases are defined as follows:

1. Reversible sorption is an essentially instantaneous attraction of bacteria to a surface. Such bacteria are held weakly near the surface, they still exhibit Brownian motion, and they are readily removed by washing the surface with 2.5°/o NaCl.

2. Irreversible sorption involves the firm adhesion of bacteria to the surface, they no longer exhibit Brownian motion, and they are not removed from the surface by washing with 2.5°/o NaCl. Irreversible sorption of bacteria from natural seawaters appears to be a selective process (Marshall, Stout and Mitchell, 1970).

Methods

Organisms. The bacteria used in this investigation were a motile Pseudomonas sp. strain R3 and a non-motile Achromobacter sp. strain R8. Both organisms were isolated from a glass surface that was immersed in natural seawater for one hr., rinsed several times in sterile 2.5°/o NaCl, plated on an artificial seawater agar, and incubated for 24 hr. at 25°C. Pseudomonas R3 requires a high salt (2.5°/o NaCl) medium, while Achromobacter R8 grown equally well in both high and low salt media.

[illegible] reversible sorption. The behaviour of the motile Pseudomonas R3 at liquid-glass interfaces was examined by preparing movie films (Kodak 4-X reversal film type 7277) of the bacteria viewed at the plane of a coverslip by phase contrast microscopy. Detailed examination of the movie films made possible a reasonable interpretation of the curious gyratory motions observed with motile bacteria at such interfaces. The maximum velocity of motile cells of Pseudomonas R3 was determined by the motility tracking method of Vaituzis and Doetsch (1969).

The effect of electrolyte concentration on reversible sorption was investigated using Achromobacter R8. As well as being non-motile, this organism grew in low salt media and could be used at very low electrolyte concentrations without causing cell lysis. R8 was grown on nutrient agar (Difco) at 25° for 24 hrs. After washing the cells twice in distilled water, aliquots of the cell suspension were mixed with equal volumes of a range of concentrations of NaCl or $MgSO_4$. Drops of the resulting suspensions were run under coverslips supported above slides by broken coverslip pieces. The preparations were allowed to stand for 30 mins. to allow those cells not sorbed at the liquid-glass interface to fall from view by gravitational force. This procedure simplified the enumeration of those cells attracted to the coverslip surface. The same technique was employed to investigate the effect of divalent cations on the reversible sorption of Pseudomonas R3.

Studies of irreversible sorption. All tests of irreversible sorption were made by immersing glass slides in an appropriate medium inoculated with Pseudomonas R3. Duplicate slides were removed at regular intervals, rinsed thoroughly with 2.5°/o NaCl, and the previously immersed area of the slide covered with a coverslip. Counts were made of cells firmly sorbed (not exhibiting Brownian motion) of at least 10 fields on both slides. All

counts are expressed as the number of cells sorbed per cm^2 of glass surface.

The media used were either 2.5°/o NaCl or an artificial seawater (ASW) as suggested by T. Waite of the following composition (g/l): NH_4Cl, 0.0007; NaCl, 24; KCl, 0.6; $MgSO_4 \cdot 7H_2O$, 5; $MgCl_2 \cdot 6H_2O$, 3.6; $CaCl_2$, 0.3; $NaNO_3$, 0.1; KH_2PO_4, 0.01; $FeCl_2$, 0.001; Tris HCl, 5.32; Tris Base, 1.97; P_{11} trace metals, 10 mls. The P_{11} trace metals was prepared as follows (g/l): disodium ethylene-diaminotetracetic acid (EDTA), 1; $FeCl_2$, 0.01; H_3BO_3, 0.2; $MnCl_2$, 0.04; $ZnCl_2$, 0.005; $CoCl_2$, 0.001. Modifications of these media are detailed in the Results section

Pseudomonas R3 was inoculated on large slopes of nutrient agar (Difco) containing 2.5°/o NaCl, and normally incubated for 24 hrs. at 25°. The bacteria were washed once in 2.5°/o NaCl before being inoculated into the test medium to give a final concentration of between 1 and 10×10^7 bacteria/ml.

For electron microscope studies of irreversibly sorbed bacteria, nickel grids (collodion-carbon films) were immersed directly in the medium containing Pseudomonas R3. The grids were removed after one hour, fixed in 2.5°/o formaldehyde in 2.5°/o NaCl (Hodgkiss and Shewan, 1968) for 30 mins., dried, rinsed in distilled water, and dried prior to shadowing with gold-palladium alloy (60°/o gold - 40°/o palladium).

Results

Motility and reversibile sorption of Pseudomonas R3

Pseudomonas R3 is a motile rod possessing a single polar flagellum (Plate 1, Fig. 1). In common with most motile bacteria, this organism exhibited a peculiar rotational behaviour at a liquid-glass interface. From a careful study of movie films of this phenomenon, these observations were made. Motile bacteria may be sorbed at the pole of the cells (an edge-to-face

attachment) and, by virtue of the motive force of the flagella, the cells rotate violently around the fixed pole (Fig. 1a). If sorbed in a face-to-face position (Fig. 1b), motile bacteria rotate in either direction in a propeller-like fashion. Motile bacteria occasionally break away from the surface, and often were found to sorb at another point on the surface. Any non-motile cells are sorbed in a face-to-face position (Fig. 1c) and show only Brownian motion. Both motile and non-motile cells were readily desorbed by washing the glass surfaces in 2.5°/c NaCl.

In view of the importance of physical forces operative between the bacterial and glass surfaces (see Discussion), an estimate of the kinetic energy of motile R3 cells was made. The maximum velocity of R3 cells was made. The maximum velocity of R3 cells was found to be 33μ/sec. Assuming an average cell mass of 10^{-12} g, then the kinetic energy of a motile cell is 16.5×10^{-18} ergs.

Effect of medium composition on irreversible sorption of Pseudomonas R3

As reported below, the irreversible sorption of R3 is affected by the presence or absence of divalent cations in artificial seawater (ASW). However, the results in Table 1 show that the deletion of divalent cations from ASW + glucose at 7 mg/l (ASWG) does not influence the numbers of bacteria initially attracted to the glass surface.

Effect of electrolyte concentration on reversible sorption of Achromobacter R3

A comparison was made of the initial attraction of cells of Achromobacter R8 in different concentrations of both monovalent and divalent electrolytes (Fig. 2). The number of cells reversibly sorbed increases as the electrolyte concentration increases or as the thickness of the electrical double layer decreases. The theoretical thickness of the diffuse double-layer (1/K) was calculated from the expression:

$$K = 0.327 \times 10^8 Z\sqrt{c}$$

where Z = valency and c = molar concentration of electrolyte. This expression holds for aqueous solutions of symmetrical electrolytes at 25° (Shaw, 1966).

Cells are reversibly sorbed at lower concentrations of a divalent electrolyte ($MgSO_4$) than of a monovalent one (NaCl), an effect clearly related to the greater compression of the double-layer in the divalent system at comparable concentrations. In both electrolyte systems, all the bacteria are repelled from the surface when the value of 1/K exceeds about 200 Å. This result suggests that the initial, reversible attraction of bacteria to a surface depends on the relative magnitude of the double-layer repulsion energy at different electrolyte concentrations in comparison with the van der Waals attraction energy.

Effect of glucose on the irreversible sorption of Pseudomonas R3

Irreversible sorption of R3 from 2.5°/o NaCl or ASW media was compared with sorption from these media enriched with 7 mg. of glucose/l. This glucose level is the equivalent of the average carbon level found in natural seawaters. Sorption was negligible from NaCl, but appreciable from ASW (Fig. 3). The addition of glucose stimulated sorption from both media. Electron microscope grids suspended in the ASW and ASWG treatments revealed the presence of very fine extracellular polymeric fibrils on bacteria sorbed from ASW (Plate 1, Fig. 2), while bacteria sorbed from ASWG showed much greater production of such fibrils (Plate 1, Fig. 3).

Since low levels of glucose stimulate sorption and polymer production by Pseudomonas R3 , the effects of higher levels of glucose were investigated. Although the growth of R3 was stimulated by higher levels of glucose (Fig. 4),

irreversible sorption of the bacteria from ASWG (30 mg/l) and ASWG (70 mg/l) was inhibited completely (Fig. 5). Sorption was rapid from ASWG (7 mg/l) and moderate from ASW. Further investigations revealed that the irreversible sorption of R3 was lowered dramatically even at glucose levels of 14 and 21 mg/l (Table 2). Flocculation of bacteria in the bulk suspension was observed in those treatments where sorption was greatest.

An examination of electron microscope grids immersed in the above treatments revealed large numbers of bacteria on those from ASWG (7 mg). All these bacteria produced extracellular polymeric fibrils. In some instances, it appeared that some sorbed bacteria had been sheared from the grid surface by washing, leaving polymer "footprints" of the bacteria on the grid surface (Plate 1, Fig. 4). Very few cells were found on grids from the ASWG (14 mg) and ASWG (21 mg) treatments. Some cells showed little evidence for polymer production (Plate 2, Fig. 5), while others appeared to produce abundant polymer (Plate 2, Fig. 6).

Although the ASW medium contains both NH_4Cl and $NaNO_3$ as nitrogen sources, investigations with the individual salts at equivalent nitrogen levels revealed that the form of nitrogen did not influence growth and the irreversible sorption of R3 to glass surfaces. Since all the nitrogen in the basic ASW medium was available to R3, then the C/N ratios ranged from 0.17 in ASWG (7 mg/l) to 1.70 in ASWG (70 mg/l). Consequently all of these media are carbon deficient, a fact that raises some questions as to the mechanism of production of extracellular polymeric materials under such conditions.

Effect of divalent cations on irreversible sorption of Pseudomonas R3

The deletion of Ca^{2+} and Mg^{2+} (with Na_2SO_4 added to provide SO_4^{2-}) from ASWG prevented the irreversible sorption of Pseudomonas R3 (Fig. 6). The

results show that the mere addition of Ca^{2+} and Mg^{2+} to 2.5°/o NaCl + glucose does not stimulate the sorption of R3 from such a medium. Total numbers of R3 in the bulk suspension remained constant in all but the ASWG (7 mg) treatment, where rapid sorption appeared to be related to the limited growth of the bacteria. Few bacteria adhered to electron microscope grids immersed in the ASWG ($-Ca^{2+}$, $-Mg^{2+}$) treatments. Those that were observed showed no evidence of polymer production (Plate 2, Figs. 7 and 8).

Although irreversible sorption is inhibited when both Ca^{2+} and Mg^{2+} are deleted from ASWG, sorption does occur when either one of the divalent cations is deleted from the medium (Table 3). The degree of sorption from the various media (Table 3) appears to be related to the growth of Pseudomonas R3 in the media (Fig. 7). Flocculation was observed consistently in the ASWG and ASWG ($-Mg^{2+}$) treatments, but not in the ASWG ($-Ca^{2+}$) and ASWG ($-Ca^{2+}$, $-Mg^{2+}$) treatments. Thus, flocculation (bacterium-bacterium interaction) in the bulk suspension does not necessarily involve the same mechanisms as sorption (bacterium-glass interaction) to a different surface.

Effect of age of inoculum on irreversible sorption of Pseudomonas R3

An attempt was made to demonstrate the requirement for active growth in bacteria for sorption by the addition of both log phase and stationary phase cells of R3 to the test media. Sorption of young (log phase) bacteria from ASWG was much faster than that of older bacteria (Fig. 8), but all the young cells were lysed. Within 24 hrs. the originally turbid bacterial suspension in ASWG had completely lysed. Neither growth, lysis, nor sorption were observed with young cells in ASWG ($-Ca^{2+}$, $-Mg^{2+}$). It is possible that lysis of young cells in ASWG may have resulted from a weakening of the cell wall during further growth of these actively metabolizing cells.

Discussion

In the sorption of marine bacteria to surfaces, different mechanisms must be involved in the essentially instantaneous reversible phase and in the time-(and probably growth) dependent irreversible phase of sorption.

Reversible sorption

The results obtained for the effects of different concentrations of monovalent and divalent electrolytes on the reversible sorption of Achromobacter R8 suggest that this phenomenon may be explained in terms of the Derjaguin-Landau and Verwey-Overbeek theory (Shaw, 1966; Weiss, 1968). This theory involves an estimation of the magnitude, and variation with interparticle distance, of the London-van der Waals attractive energies between two surfaces and the electrical repulsive energies resulting from the overlapping ionic atmospheres (diffuse double-layers) around the surfaces.

The energies of interaction between cells of Pseudomonas R3 and a glass surface at different values for the double-layer thickness have been computed by Dr. James Harlos and Dr. Leonard Weiss, Dept. of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, N.Y. This was done with a programme based on a rearranged version of the formula derived by Hogg, Healy and Fuerstenan (1966):

$$V_T = V_R + V_A$$

where V_R = repulsion energy

$$= \frac{\epsilon}{4}\left(\frac{a_1 a_2}{a_1+a_2}\right)\left[(\psi_1+\psi_2)^2 \ln(1+e^{-KH}) + (\psi_1-\psi_2)^2 \ln(1-e^{-KH})\right]$$

V_A = attraction energy

$$= -\frac{A}{6}\left(\frac{a_1 a_2}{a_1+a_2}\right)\frac{1}{H}$$

where a is the radius of curvature of the particle, $a_1 = 10^5$ Å (glass) and $a_2 = 0.4\mu$ (bacterium); ψ is the surface potential of a particle, $\psi_1 = -15$mV (glass), $\psi_2 = -25$mV (bacterium); K is the inverse Debye-Huckel length, 1/K varies from 6.85 Å at 2×10^{-1} M NaCl and 200 Å at 2×10^{-4} M NaCl; H is the distance of closest approach of the 2 particles; ϵ is the dielectric constant (of water); A is Hamaker's constant (using values of 5×10^{-14} and 5×10^{-15} ergs).

The series of curves in Fig. 9 demonstrate the increase in magnitude of the resultant repulsion curves and the increase in particle separation with decreasing electrolyte concentration (increasing values of 1/K). At high electrolyte concentrations, a secondary minimum ("attractive trough") is apparent. The progressive increase in repulsion energy with decreasing electrolyte concentration closely parallels the observed decrease in reversible sorption of Achromobacter R8 (Fig. 2). The extent of the repulsion barrier at a value of 1/K of 200 Å is shown in Fig. 10 for two different values of A.

In the reversible phase of sorption in seawater or 2.5°/o NaCl it is likely that cells of R3 are attracted to the point of the secondary minimum, a small but finite distance from the glass surface. It is unlikely that thermal motion would provide sufficient energy for the bacteria to overcome the repulsion barrier. In fact, the kinetic energy of motile cells of Pseudomonas R3 (16.5×10^{-18} ergs) is not sufficient to overcome the repulsion barrier at any value for 1/K shown in Fig. 9. The magnitude of the attractive energy at the secondary minimum may not be sufficient to hold the bacteria against the shearing effect of the rinsing process. The movie films clearly showed that motile cells of Pseudomonas R3 can break away from the surface, although the attractive energy does hold such cells against violent rotational movements resulting from flagellar activity.

Irreversible sorption

The irreversible phase of sorption implies a firmer adhesion of bacteria to a surface. Polymeric bridging between the bacterial surface and that of the test surface might provide a mechanism for overcoming the repulsion barrier between such surfaces. Such a mechanism has been proposed for the adhesion of tissue cells (Moscona, 1962) and sponge cells (Humphreys, 1965), and for the flocculation of microbial cells (Tenney and Stumm, 1965; Busch and Stumm, 1968). The electron micrographs presented provide evidence for the production of polymeric fibrils by Pseudomonas R3. The nature of the polymeric material has not been investigated. Where the bacteria are unable to grow, as in the ASWG ($-Ca^{2+}$, $-Mg^{2+}$) medium, polymer was not detected and irreversible sorption did not occur. The observation that polymer "footprints" are left behind when cells are sheared from the surface suggests that this polymer is more tightly bound to the glass surface than to the bacterial cell. This may result from the polymer bridging the repulsion barrier and becoming firmly chemisorbed to the glass surface.

The fact that extremely low levels of available carbon stimulated irreversible sorption while higher levels inhibited this process may be very important in terms of microbial ecology. In natural seawaters the available carbon levels are usually very low, and such conditions probably favour the firm adhesion of microorganisms to surfaces immersed in such environments. The reason for these effects of different glucose levels is not clear. During the limited growth of Pseudomonas R3 at low carbon levels the cells may become "leaky" and may extrude internal macromolecular material. The lack of sorption of bacteria at higher glucose levels might indicate that the cells are more normal (less leaky). When the bacteria cannot grow, as

in ASWG (+Ca^{2+}, - Mg^{2+}) medium, they may not develop leakiness and polymer may not be produced. This possibility is supported by the observation that young (log phase) cells in such do not lyse, while similar bacteria in complete ASWG medium rapidly lyse.

The observed effects of deletion of divalent cations from the complete ASWG medium suggest that the lack of sorption when both Ca^{2+} and Mg^{2+} are lacking is related to a lack of growth of Pseudomonas R3. When only one of the divalent cations was deleted, growth occurred and was related to the degree of irreversible sorption.

Other factors are probably involved in both phases of sorption of bacteria to surfaces. The sorption to a surface of monolayers of various macromolecules present in seawater or even in bacterial cultures might drastically alter such physical characteristics of the surface as wettability and surface charge properties (Baier, Shafrin and Zisman, 1968).

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Explanation of Plates

All figures are electron micrographs of *Pseudomonas* sp. strain R3.

Plate 1

Figure 1. Single polar flagellum of R3.

Figure 2. Extracellular polymeric fibrils on bacteria sorbed on grid immersed in artificial seawater.

Figure 3. As in Figure 2, but grid immersed in artificial seawater + glucose (7 mg/l).

Figure 4. Polymer "footprints" remaining after bacteria were sheared from the grid surface.

PLATE I

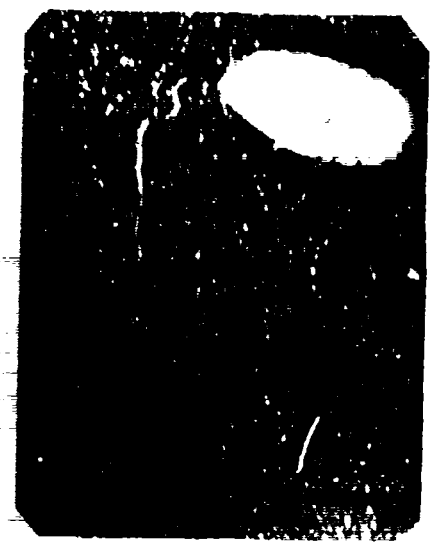

Fig. 1

Fig. 2

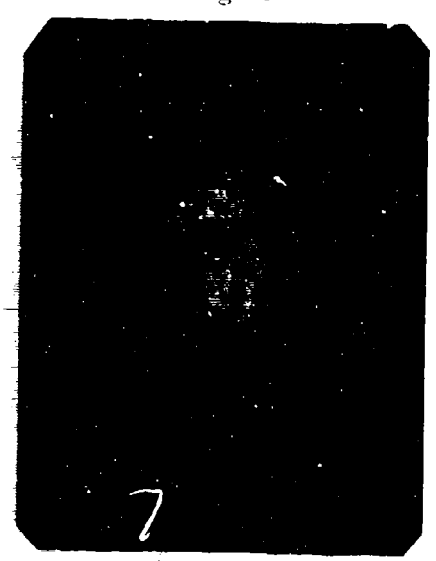

Fig. 3

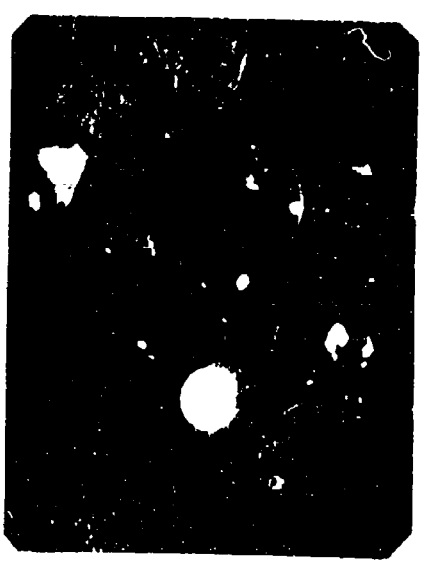

Fig. 4

Plate 2

Figure 5. Grid immersed in artificial seawater + glucose (14 mg/l). Bacterium showing no extracellular polymer production.

Figure 6. As in Figure 5, but bacteria showing abundant polymer production.

Figure 7. Bacterium lacking extracellular polymeric fibrils. From ASWG without Ca^{2+} and Mg^{2+}.

Figure 8. As in Figure 7.

PLATE II

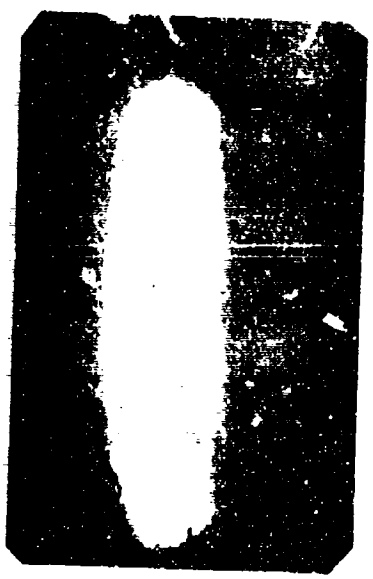

Fig. 7

Table 1. Effect of divalent cations in artificial seawater on the reversible sorption of Pseudomonas R3

Medium	No. bacteria sorbed/cm^2
ASWG	5.35×10^3
ASWG ($-Ca^{2+}$)	5.27×10^3
ASWG ($-Mg^{2+}$)	5.58×10^3
ASWG ($-Ca^{2+}$, $-Mg^{2+}$)	5.42×10^3

Table 2. Effect of glucose levels in artificial seawater on growth and irreversible sorption of Pseudomonas R3

Glucose (mg/l)	No. bacteria/ml. after 24 hrs.*	No. bacteria sorbed/cm^2 (x 10^3) 20 hr	4 days
0	14.8×10^7 †	44.6	488
7	26.1×10^7 †	65.8	514
14	33.2×10^7	2.3	78
21	44.1×10^7	2.1	31

* Initial inoculum = 10.0×10^7 bacteria/ml

† Flocculation evident in the bulk suspension

Table 3. Effect of divalent cations in artificial seawater on the irreversible sorption of Pseudomonas R3

	no. bacteria sorbed / cm^2	
Medium	24-hour	48-hour
ASWG	242×10^3*	464×10^3*
ASWG ($-Ca^{2+}$)	155×10^3*	400×10^3*
ASWG ($-Mg^{2+}$)	126×10^3*	280×10^3*
ASWG ($-Ca^{2+}$, $-Mg^{2+}$)	0	0

* Flocculation evident in the bulk suspension.

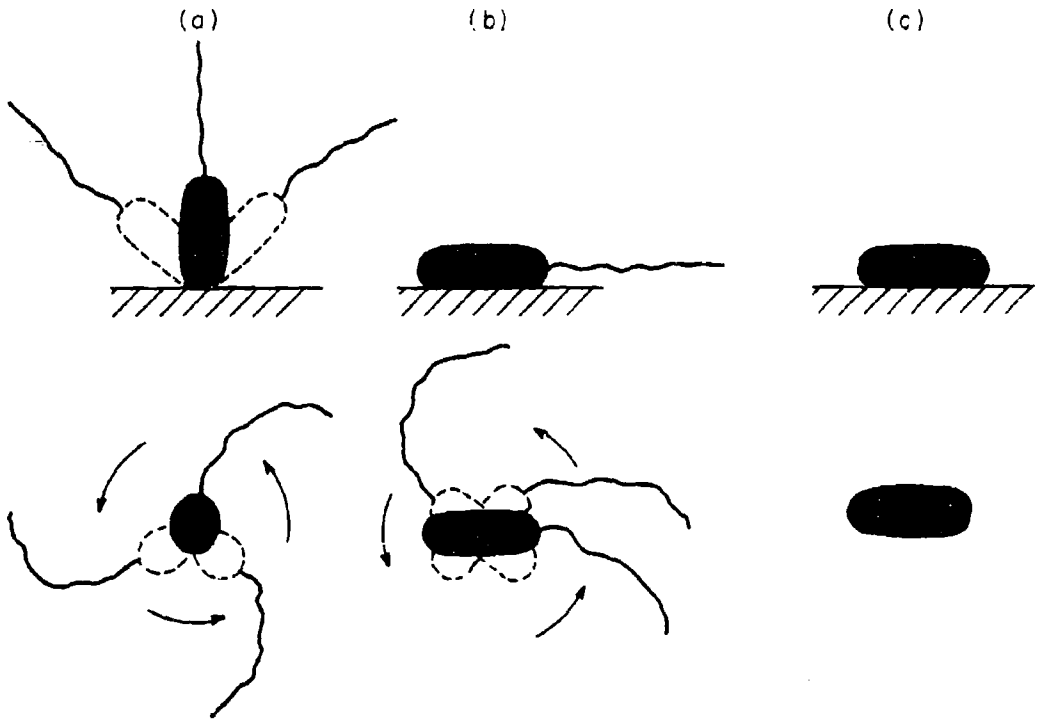

Figure 1. A diagrammatic interpretation of the reversible sorption of Pseudomonas R3 to a glass surface: (a) and (b) illustrate the rotational movements of motile bacteria in an edge-to-face and a face-to-face manner, respectively; (c) face-to-face sorption of non-motile bacteria.

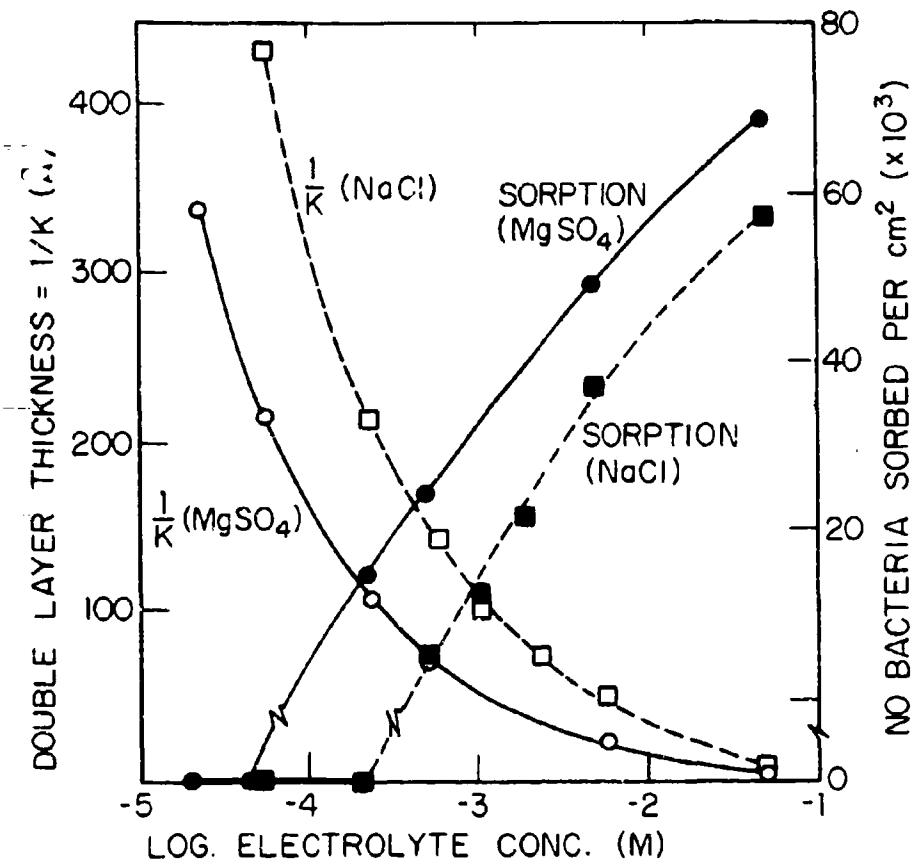

Figure 2. Reversible sorption of Achromobacter R8 and the theoretical double-layer thickness ($\frac{1}{K}$) in relation to electrolyte concentration and valency. □—□, $\frac{1}{K}$ for NaCl; ○—○, $\frac{1}{K}$ for $MgSO_4$; ■—■, sorption of R8 from NaCl; ●—●, sorption of R_8 from $MgSO_4$.

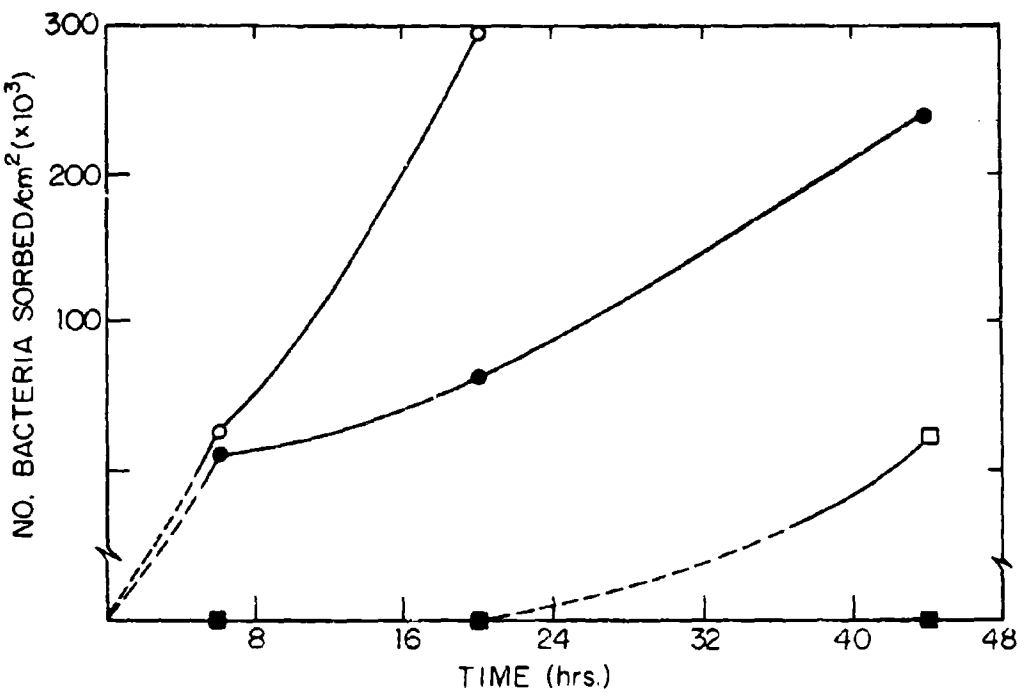

Figure 3. Irreversible sorption of Pseudomonas R3 from NaCl and artificial seawater (ASW). ■—■, 2.5% NaCl; □—□ 2.5% NaCl + glucose (7 mg/ l); ○—○, ASW + glucose (7 mg/ l). ●—●, ASW.

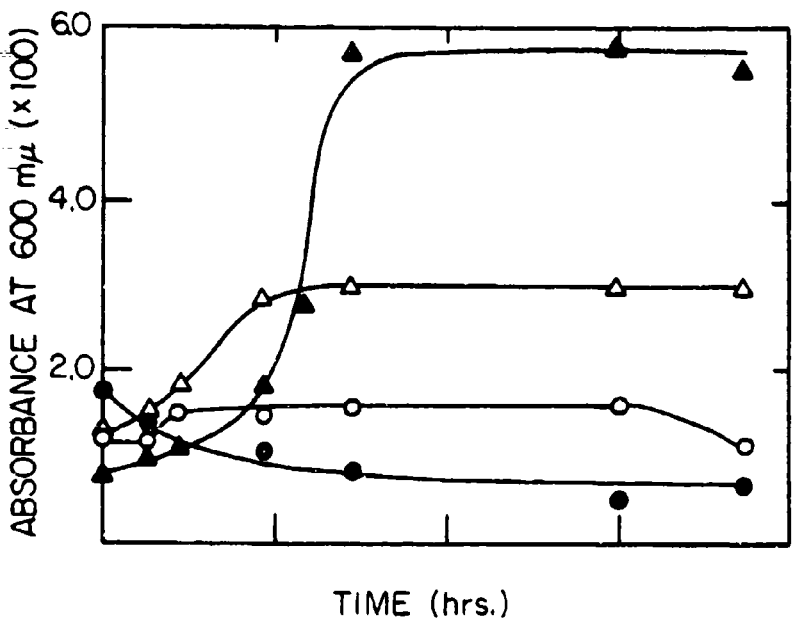

Figure 4. Effect of glucose concentration on the multiplication of Pseudomonas R3 in ASW. ●—●, ASW; ○—○, ASW + glucose (7 mg/l); △—△, ASW + glucose (30 mg/l); ▲—▲, ASW + glucose (70 mg/l).

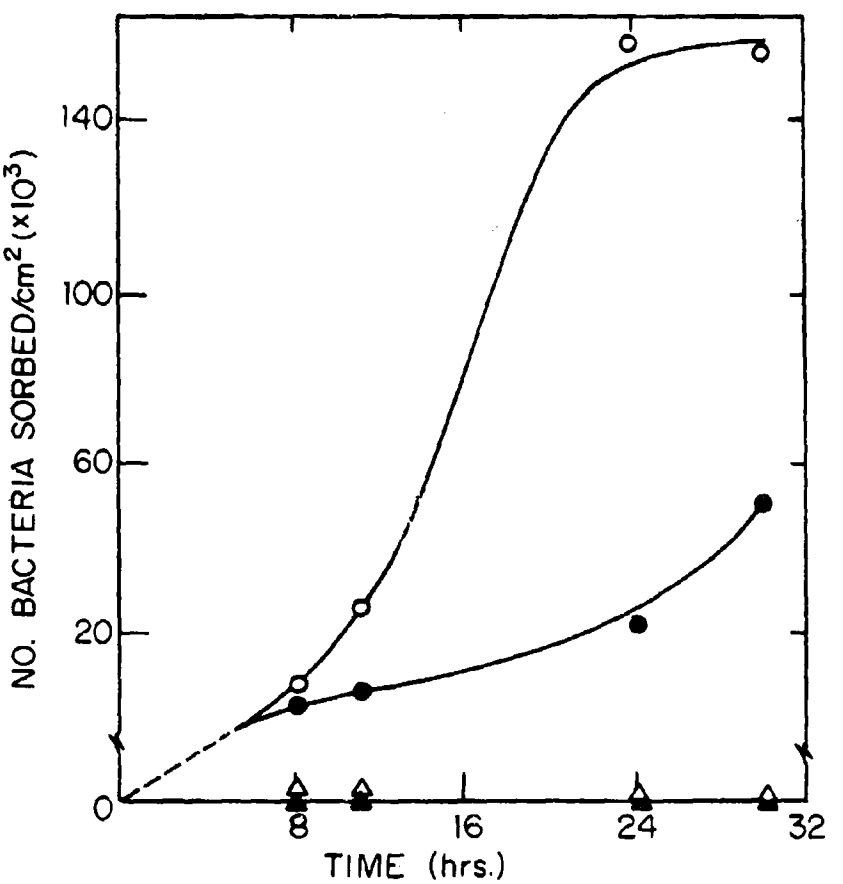

Figure 5. Effect of glucose concentration on the irreversible sorption of Pseudomonas R3 from ASW. Symbols as in Figure 4.

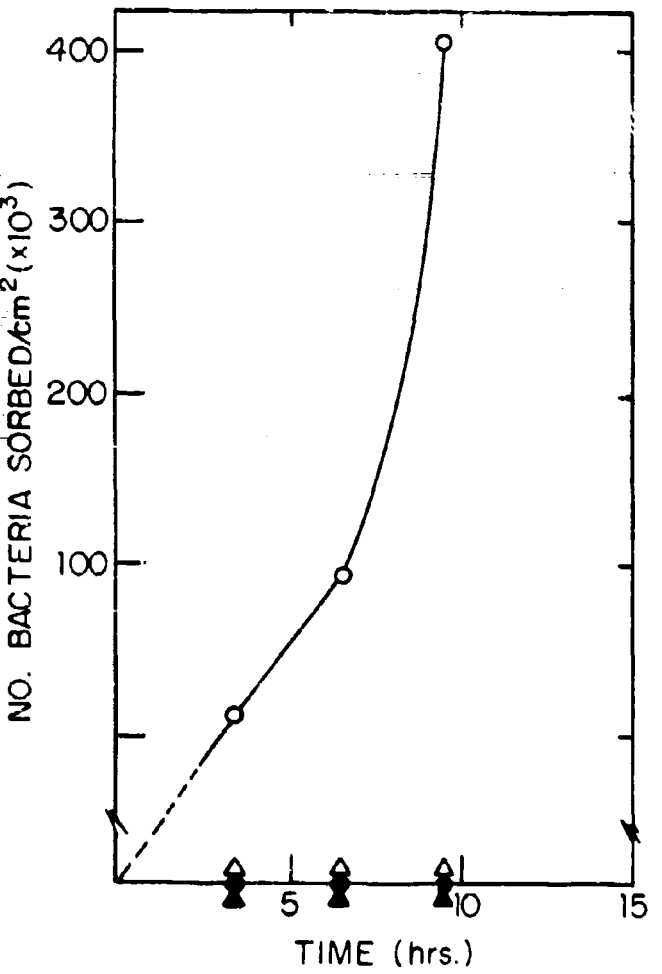

Figure 6. Effect of divalent cations on the irreversible sorption of Pseudomonas R3 from NaCl and ASW. ▲—▲, 2.5% NaCl + glucose; △—△, 2.5% NaCl + glucose (+ Ca^{2+}, + Mg^{2+}); ○—○, ASW + glucose; ●—●, ASW + glucose (-C^{2+}, -Mg^{2+}).

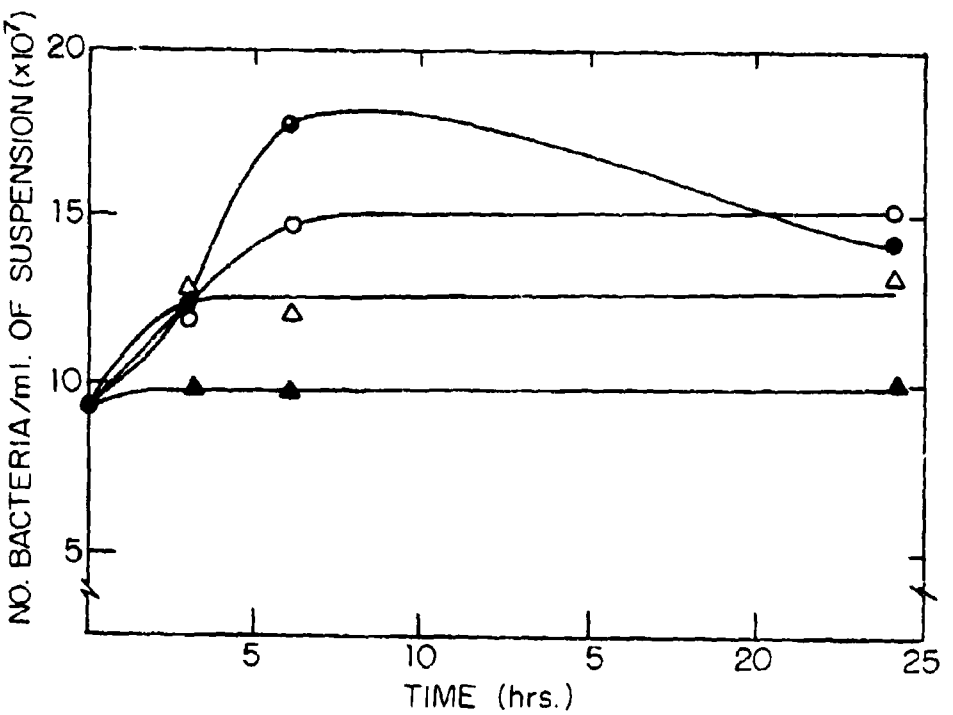

Figure 7. Effect of divalent cations on growth of Pseudomonas R3 in ASWG (glucose at 7 mg/ l). ●—●, ASWG; ○—○, ASWG (-Ca^{2+}); △—△, ASWG (-Mg^{2+}); ▲—▲, ASWG (-Ca^{2+}, -Mg^{2+}).

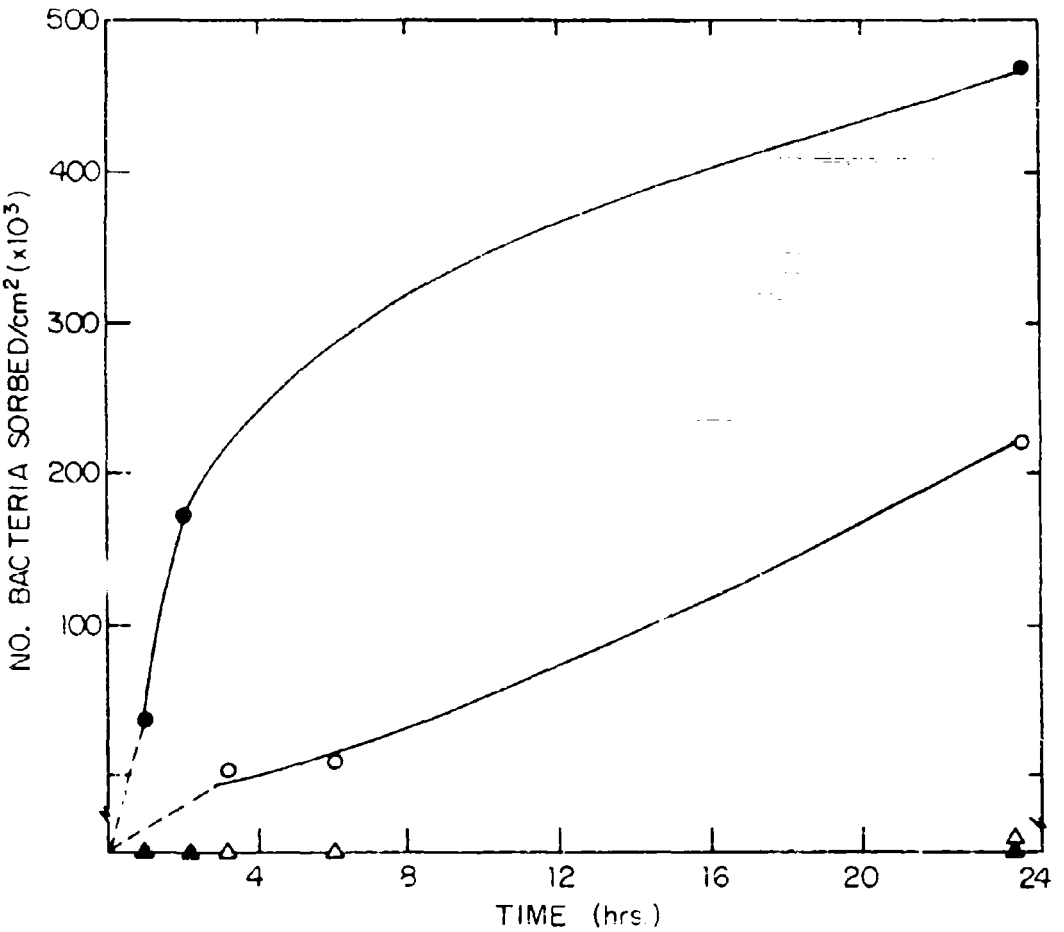

Figure 8. Effect of age of inoculum on reversible sorption of Pseudomonas R3 from ASWG. ●—● , log. phase bacteria in ASWG, all bacteria lysed; ▲—▲ , log. phase bacteria in ASWG (-Ca^{2+}, -Mg^{2+}), no lysis, ○—○ , stationary phase bacteria in ASWG, no lysis; △—△ , stationary phase bacteria in ASWG (-Ca^{2+}, Mg^{2+}), no lysis.

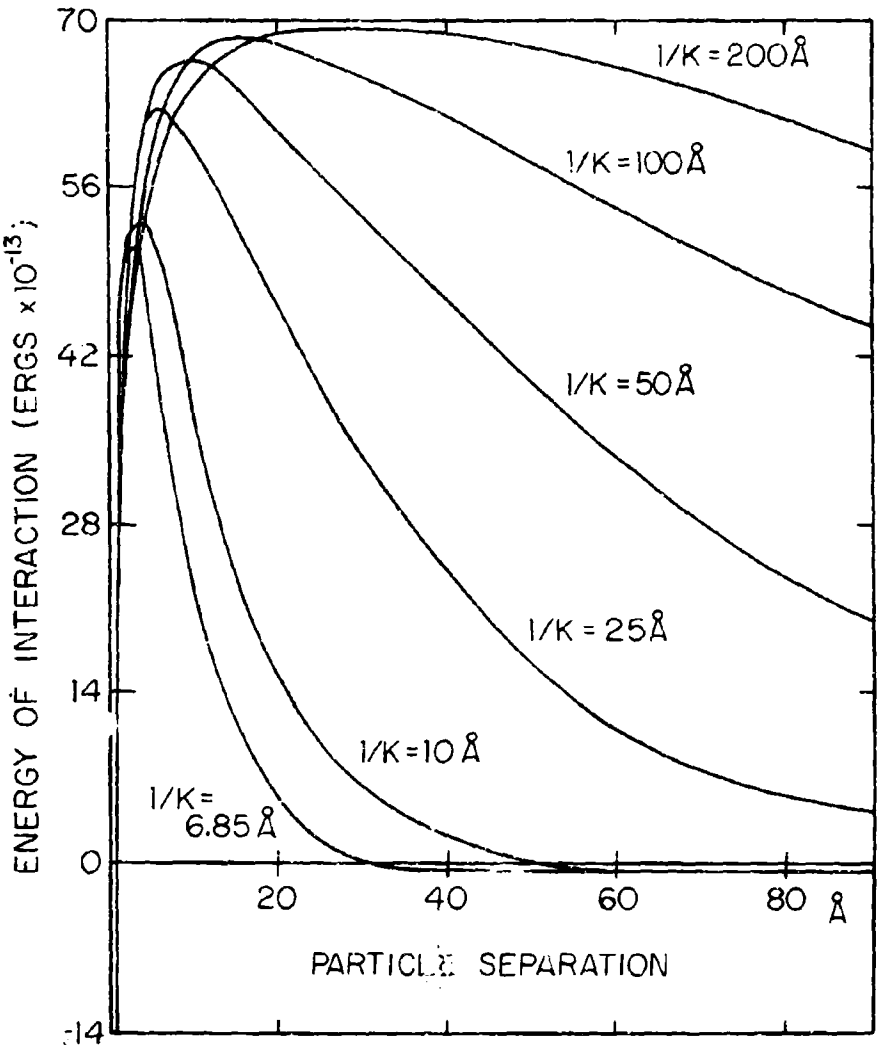

Figure 9. Computed curves of the energy of interaction between glass and bacterial (Achromobacter R8) surfaces at varying electrolyte concentrations (varying double-layer repulsion) using a value for A of 5×10^{-15} ergs.

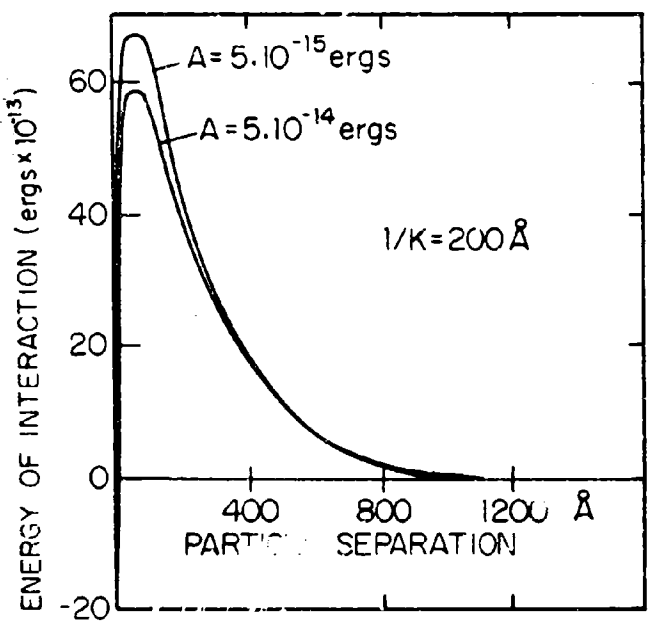

Figure 10. Energy of interaction between glass and bacterial (Achromobacter R8) surfaces for different values of the Hamaker constant (A) when $\frac{1}{K}$ = 200 Å. Top curve, A 4 ^{15} ergs; bottom curve, A = 5 x 10^{-14} ergs.

References

Baier, R. E., Shafrin, E. G. and Zisman, W. A. (1968). Adhesion: Mechanisms that assist or impede it. Science, 162, 1360.

Busch, P. L. and Stumm, W. (1969). Chemical interactions in the aggregation of bacteria bioflocculation in waste treatment. Environmental Science and Technology, 2, 49.

Corpe, W. A. (1970a) An acid polysaccharide produced by a primary film-forming marine bacterium. Developments in Industrial Microbiology, 11, 402.

Corpe, W. A. (1970b). Attachment of marine bacteria to solid surfaces. In Biological Adhesions. Ed. by Manly. New York: Academic Press.

Hodgkiss, W. and Shewan, J. M. (1968). Problems and modern principles in the taxonomy of marine bacteria. Advances in Microbiology of the Sea, 1, 127.

Hogg, Healy, and Fuerstenan, (1966).

Transactions of the Faraday Society, 62, 1638.

Humphreys, T. (1965). Cell surface components participating in aggregation: Evidence for a new cell particulate. Experimental Cell Research, 40, 539.

Marshall, K. C. (1970). Sorptive interactions between soil particles and microorganisms. In Soil Biochemistry, Volume II. Ed. by A. D. McLaren and J. J. Skujins. New York: Marcel Dekker, Inc. (in press).

Marshall, K. C., Stout, R. and Mitchell, R. (1970). Selective sorption of bacteria from seawater. Journal of General Microbiology (submitted for publication).

Moscona, A. A. (1962). Analysis of cell recombinations in experimental synthesis of tissues, in vitro. Journal of Cellular and Comparative Physiology, 60, 65.

Shaw, D. J. (1966). Introduction to Colloid and Surface Chemistry. London: Butterworth and Co.

Tenney, M. W. and Stumm, W. (1965). Chemical flocculation of microorganisms in biological waste treatment. Journal of the Water Pollution Control Federation, 37, 1370.

Vaituzis, A. and Doetsch, R. N. (1969). Motility tracks: Technique for quantitative study of bacterial movement. Applied Microbiology, 17, 584 (1969).

Weiss, L. (1968). Studies on cellular adhesion in tissue-culture X. An experimental and theoretical approach to interaction forces between cells and glass. Experimental Cell Research, 53, 603.

Wood, E. J. F. (1967). Microbiology of Oceans and Estuaries. Amsterdam. Elsevier Publishing Co.

ZoBell, C. E. (1943). Journal of Bacteriology, 46, 39.

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I. SELECTIVE SORPTION OF BACTERIA FROM SEAWATER
II. MECHANISM OF THE INITIAL EVENTS IN THE SORPTION OF MARINE BACTERIA TO SURFACES

Interim technical report

K. C. Marshall, Ruby Stout and R. Mitchell

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SUPPLEMENTARY NOTES

Office of Naval Research

ABSTRACT

In Part I a distinct sequence of sorption of different bacterial types has been observed both on glass slides and electron microscope grids immersed in seawater for periods of up to 24 hours. A comparison of the bacterial groups initially attracted to a surface with those subsequently adhering firmly to the surface suggests a selective irreversible sorption of certain groups of marine bacteria. Firm adhesion of bacteria in the short time periods considered does not involve pili or holdfast structures. The ability to produce extracellular polymeric fibrils may be important in such selective sorption.

In Part II two phases in the process of sorption of marine bacteria to surfaces have been defined as (a) an instantaneous reversible phase, and (b) a time-dependent irreversible phase.

Reversible sorption of the non-motile Achromobacter sp. strain R8 decreases to zero as the electrolyte concentration decreases, or as the thickness of the electrical double-layer increases. The electrolyte concentration at which all bacteria are repelled from the glass surface depends on the valency of the electrolyte. The reversible phase of sorption is interpreted in terms of the balance between the electrical double-layer repulsion energies at different electrolyte concentrations and the van der Waals attractive energies. Even at the electrolyte concentration of seawater, the bacteria probably are held at a small but finite distance from the glass surface by a repulsion barrier. The rotational motion of the motile Pseudomonas sp. strain R3 at a liquid-glass interface is considered in terms of the concept of reversible sorption.

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Selective Sorption Initial Events in Bacterial Sorption Mechanism of Bacterial Sorption Sorption of Bacteria to Surfaces						